DETECTION OF VIABLE BACTERIA CELLS BY BIOLUMINESCENCE: A BIOENERGETIC APPROACH[1]

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ABSTRACT

The cellular ATP content of fourteen freshly harvested bacteria including Bacillus, Campylobacter, Citrobacter, Escherichia, Lactobacillus, Listeria, Pediococcus, Pseudomonas, Salmonella, Streptococcus *and* Yersinia, *was determined using a luciferin-luciferase bioluminescence approach. Incubation of bacteria with carbonyl cyanide meta-chlorophenyl hydrazone (CCCP), a membrane protonophore, prior to cell breakage substantially lowered the bioluminescence signals indicating a decrease of cellular ATP content. The addition of CCCP after cell breakage had no detectable effect on the ATP levels. This differential effect of CCCP was not observed using heat-killed bacteria, i.e., the ATP content was not affected by CCCP incubation. The CCCP effects on cellular ATP level were detectable in bacterial suspensions with concentrations ranging from 10^6 to 10^3 CFU/mL. Upon cold storage, the ATP content, but not the population of viable bacteria, decreased. The ATP content could be partially restored by the addition of glucose. The ATP content restored by the addition of glucose was also sensitive to CCCP treatment. These results demonstrated that viable bacterial cells can be differentiated from dead cells by their responses to membrane protonophores.*

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INTRODUCTION

The presence of pathogenic bacteria in foods is a serious concern to public health. The Center for Disease Control (CDC) estimates that 76 million people get sick, more than 300,000 are hospitalized, and 5,000 Americans die each year from foodborne illnesses (Center for Disease Control and Prevention 2002). To facilitate the detection of pathogens, many new and highly sensitive biosensor-based methodologies have also been developed to complement or replace traditional culture methods (Hall 2002; Loung *et al.* 1997; Swaminathan and Feng 1994). However, many new methods, including PCR, do not yield any information on the viability of the bacteria found in foods. Although the viability of suspected bacteria may be established by cell-culture approach, it is desirable to develop alternative approaches that may be incorporated in the detection process.

Activities associated with cellular metabolites such as ATP have been utilized directly and indirectly to qualitatively indicate the presence of live microbes including bacteria in foods and food processing facilities (Knowles 1977; Siragusa *et al.* 1996; Sharpe *et al.* 1970). Similarly, cellular NAD(P)H has also been chosen as a target metabolite to indicate the presence of viable cells (De Graef *et al.* 1999; Yamashoji and Takeda 2001). Both ATP (Aflalo and DeLuca 1987; Strehler and Totter 1954) and NAD(P)H (Yamashoji *et al.* 2001) may be determined by luminescence techniques. In the case of ATP determination, the cells are usually lysed to release the celluar ATP. Externally-added luciferin-luciferase system then interacts with ATP to emit luminescence. On the other hand, a membrane permeable quinone is used to shunt the reducing power from NAD(P)H to reduce molecular oxygen to active oxygen species (O_2^- and H_2O_2) that can be measured by luminescence emitted from coupled oxidation of externally-added luminol. Consequently, there is no need to lyse the intact cells for NAD(P)H determination.

The cellular concentrations of ATP and NAD(P)H are regulated by the bio-energetic status of the cells, i.e., availability of carbon substrates, oxygen, etc. This dependence adds an uncertainty to relate luminescence to the concentration of viable cells. One may easily underestimate microbe concentration by the low luminescence originating from relatively large number of nutrient-deprived cells. Despite this complication in quantitative determination, the luminescence approaches for ATP (Siragusa *et al.* 1996) and NAD(P)H (Yamashoji and Takeda 2001) have been successfully used to detect $\sim 10^3$ to 10^4 CFU/mL of freshly cultured *E. coli* cells.

Recently, we have developed a new approach to ascertain the presence of viable *E. coli* O157:H7 in ground beef by an ATP-bioluminescence method (Tu *et al.* 1999, 2000). In this development, *E. coli* O157 specific immunomagnetic beads were applied to capture the bacteria prior to lysis by commercially

available reagents to release cellular ATP for measurement via luciferin-luciferase induced luminescence. The bioenergetic status of the bacteria was adjusted by the addition of glucose, a carbon energy source, and carbonyl cyanide meta-chlorophenyl hydrazone (CCCP), a membrane protonophore. Cold storage decreased both the rate of oxygen consumption and the ATP content of the bacteria. However, the decreases could be partially restored by the addition of glucose at the end of the storage. On the other hand, the presence of CCCP enhanced the oxygen consumption and medium acidification but significantly decreased the ATP content of viable bacteria. None of the glucose and CCCP effects could be detected with heat-killed and γ-ray irradiated *E. coli* O157:H7. Thus, immunomagnetic capture of the *E. coli* followed by testing the bioenergetic responses of captured bacteria could ascertain the presence of viable *E. coli* O157:H7. This CCCP effect allowed the detection of less than one CFU of the *E. coli* per gram of ground beef after enrichment for 6 h at 37C.

Potentially, this method may be extended to detect the presence of other viable pathogenic bacteria if suitable antibodies are available for producing specific immunomagnetic beads and the described CCCP effects are common to other bacteria. To determine the potential applicability of the CCCP effects (Tu *et al.* 1999) in other bacteria was the primary objective of the current study. We randomly chose 14 different strains of bacteria (11 species from 10 genera) and found that the CCCP treatment caused decreases in cellular ATP content of viable cells of all tested bacteria. These results enhance the attractiveness of applying protonophores coupled with ATP measurements to ascertain the presence of viable bacteria.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

Cultures of various strains of different types of bacteria were added to suitable growth media and incubated at appropriate temperatures for 18 to 36 h. All bacterial strains and culture conditions are listed in Table 1. At the end of incubation, the cell density (CFU/mL) was determined by standard plate culture and counting techniques. All cultures were grown for 18-36 h in 25 mL media in a 50 mL flask. Stationary phase bacteria were harvested by centrifugation and then suspended in a buffer containing 10 mM Tris, pH 7.5, 2.5 mM Mg SO_4 and 150 mM NaCl (TBS). To determine CFU/mL, each culture was diluted in TBS to $\sim 10^3$ cells/mL, and two-50 μL aliquots were spread onto the appropriate media. The plates were incubated overnight and the resulting colonies were counted. To prepare heat-killed cells, bacterial samples (10^7 CFU/mL) in TBS were heated for 10 min at 100C in a dry sand bath.

Cellular ATP Determination

The intensity of the bioluminescence catalyzed by luciferase was used to estimate the ATP content in bacterial cells. Bacterial samples were treated with 50% (v/v) of B-PER (bacterial protein extraction reagent), a cell lysis reagent specific to bacteria. The released cellular ATP was determined from the luminescence emitted from luciferase catalyzed oxidation of luciferin. A Berthold FB12 luminometer was used to record the intensity of the luminescence.

Effects of CCCP

To test the effects of CCCP on cellular ATP content, $\sim 10^6$ live or heat-killed bacterial cells in 100 μL of TBS were lysed by B-PER at room temperature for 10 min prior to the incubation with 2 μg of CCCP for 30 min at 37C. At the end of incubation, 100 μL of luciferin-luciferase mixture was added and the luminescence was recorded. For a second set of bacterial samples, the cells were first incubated with 2 μg of CCCP for 30 min at 37C prior to being lysed by B-PER at room temperature for 10 min. The ATP content was similarly measured by the addition of luciferin-luciferase. A decreased ATP content in the cells treated with CCCP prior to lysis is an indicator of cell viability.

Effects of Glucose

Freshly cultured bacterial cells were washed and resuspended in 1 mL TBS buffer in microfuge tubes. One set of tubes was refrigerated and cells from a second set were diluted 100-fold using TBS (with and without 10 mM glucose) and incubated for 1 h at 37C shaking at 250 rpm. Aliquots of 100 μL of the bacterial cell suspensions (3 samples per treatment) were used to determine the total and CCCP-induced decrease in ATP content of the bacteria. The same measurements were repeated using the bacterial cultures after cold-storaged for 24 and 72 h in TBS. The concentration of viable bacteria was determined for all samples using BHIA plates.

Materials

Firefly extract containing luciferin-luciferase was a product of Fluka-Sigma (Cat #62646). B-PER was purchased from Pierce (Cat #78248). All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Bioenergetic Consideration of CCCP Effects

The cellular level of ATP in viable cells at steady state is determined by the rates of ATP synthesis and utilization. It is known that the majority of cellular ATP synthesis is associated with the membrane electron transfer process. It is generally agreed that the vectorial membrane proton transport serves as the key intermediate to drive electron transfer-related ATP synthesis (Mitchell 1975) and energy-dependent solute transport (Epstein 1990; Scarpa *et al.* 1992). Cells utilize the energy released from ATP hydrolysis by membrane H^+-ATPases to actively maintain the internal concentration of ions and small metabolites through the actions of membrane transport systems (Carafoli and Scarpa 1982). Being a membrane protonophore, CCCP has the ability to discharge the proton gradient needed to support ATP synthesis and membrane transport. Consequently, its presence decreases the ATP synthesis by diminishing the proton electrochemical potential ($\Delta\mu_{H+}$) and increases the ATP utilization as the cells are forced to hydrolyze more ATP in an attempt to recover the proton potential. The action of CCCP and its bioenergetic effects are illustrated in Fig. 1.

Verification of CCCP Effects

Indeed, the predicted CCCP effects on the cellular ATP level, were verified in our previous studies of *E. coli* O157:H7 (Tu *et al.* 1999) and shown to be a measure of cell viability. In the current study, we have expanded the tests on CCCP effects as an indicator of cell viability to those bacteria listed in Table 1. Both freshly harvested and heat-killed bacteria were used. The results are summarized in Fig. 2. For freshly cultured bacteria, the cellular content of ATP (measured by cell lysis prior to CCCP addition) was substantially decreased by the actions of CCCP (measured by CCCP addition prior to cell lysis). Although heat treatment of the cells decreased the cellular ATP content by ~60 to 90%, no further decrease in ATP content were induced by CCCP treatment of heat-killed cells. Since all 14 tested strains of bacteria showed a similar response to the CCCP treatment, this approach may have the potential to be a general indicator to ascertain the presence of viable bacteria.

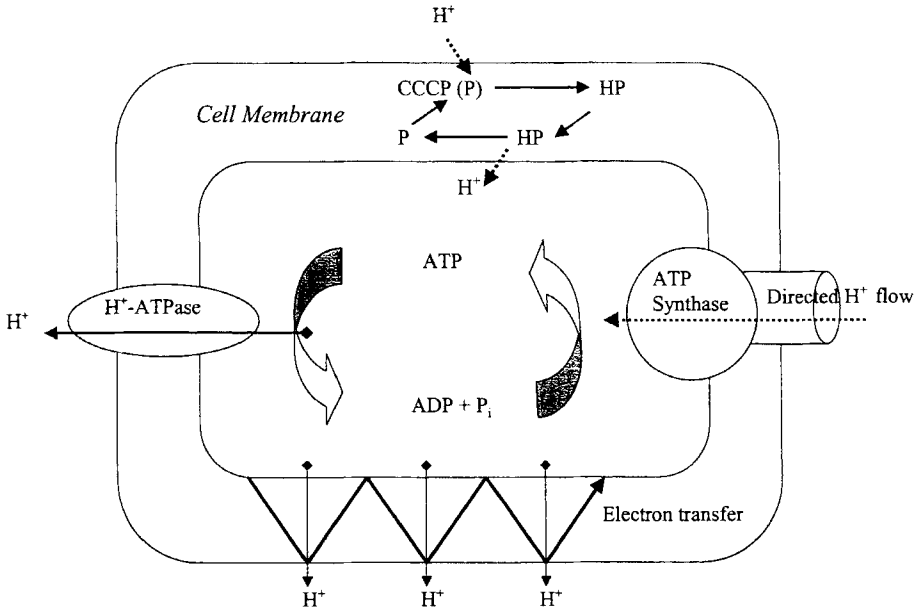

FIG. 1. BIOENERGETIC EFFECTS OF PROTONOPHORE

In viable cells, electron transfer process generates energized protons to support ATP synthesis by membrane ATP synthase. Part of the ATP is utilized to pump protons to the cell exterior. The proton pumping actions of the ATPase and the electron transfer process maintains a proton electrochemical potential ($\Delta\mu_H{}^+$) that contains a membrane potential, $\Delta\psi$ (negative inside) and a proton gradient, ΔpH (acidic outside). Both the membrane potential and the pH gradient are used to support secondary transport processes. Being a hydropholic weak-acid, protonophores such as CCCP, can pick up protons from acidic outside medium and release the protons to the alkaline interior. The proton shuttling by CCCP results in a collapse of the membrane potential and the proton gradient. This collapse also removes the metabolic feed-control of the ATPase activity. Consequently, the presence of protonophores decreases the cellular ATP content by accelerating ATP hydrolysis and inhibiting ATP synthesis.

Sensitivity of CCCP Treatment to Determine Viable Bacteria

The effects of CCCP shown in Fig. 3 indicated that the ATP level of viable bacteria is decreased by CCCP as predicted from its effects on the movement of protons in living cells. The sensitivity of this approach, under applied conditions, was evaluated using serially diluted bacterial suspensions of *Listeria monocytogenes, Salmonella typhimurium, Bacillus cereus* and *Pseudomonas putida* at concentration of 10^5 to 10^2 CFU/mL. The CCCP induced decreases of cellular ATP were determined and plotted against the bacterial concentration. CCCP induced ATP decrease is observed even at 10^2 CFU/mL of all bacteria

TABLE 1.
GROWTH CONDITIONS FOR TESTED BACTERIA

	Escherichia coli	*Salmonella typhimurium*	*Bacillus cereus*	*Pseudomonas putida*	*Pediococcus acidilactici*	*Lactobacillus bulgaricus*	*Citrobacter freundii*	*Yersinia entercolitica*	*Listeria monocytogenes*	*Campylobacter jejuni/coli*
Strains	K-12 O157:H7 (B1409, 933)	G8430	ATCC 49046	KT2442	F	ATCC 11842	ATCC 33128	O:TAC-C	FSL N1-227 ATCC 19115	ATCC 33560/ ATCC 33559
Gram	Negative	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Positive	Negative
Shape	Rod	Rod	Rod	Rod	Cocci	Rod	Rod	Rod	Rod	Spiral
Medium	BHI[a]	NB[b]	NB	NB	MRS[d]	MRS	NB	NB	BHI	MH[c]
Temp (°C)	37	37	30	37	30	37	37	37	37	42
Time (h)	18	18	24	18	24	18	18	18	18	36
Aeration[e]	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	*

[a]Brain heart infusion medium. [b]Nutrient broth. [c]Mueller Hinton. [d]De Man, Rogosa and Sharpe medium. [e]Yes-aeration of incubation media achieved by rotating the sample-flasks at 160 rpm; No-stationary cultures; *microaerophilic growth in BBL GasPak jar using CampyPak system.

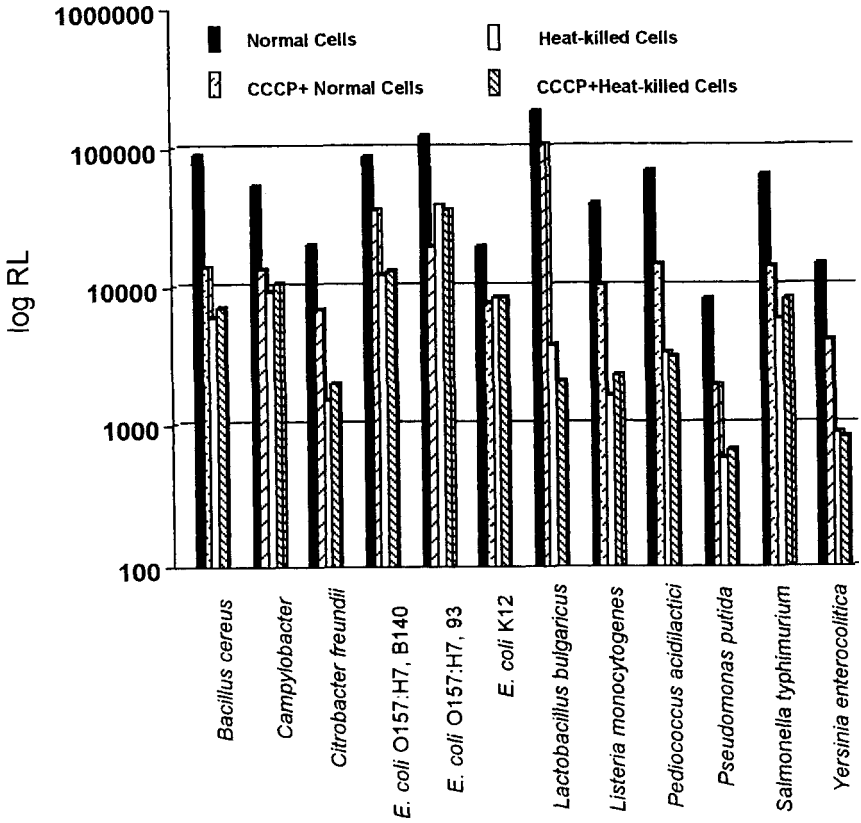

FIG. 2. EFFECTS OF CCCP ON VIABLE AND HEAT-KILLED BACTERIA
The normal ATP content of freshly cultured and heat-killed bacteria were determined as described in the text. To test the effects of CCCP, the bacteria were incubated with the protonophore prior to the lysis by B-PER. The addition of CCCP after cell lysis using B-PER provided a control for the possible quenching of the bioluminescence. The luminescence intensity related to ATP content was expressed as relative luminescence unit (RLU) measured by the luminometer described in Material and Methods. The data shown represent an average of 3 independent measurements with an error range of ± 10%.

tested ($\Delta ATP > 0$). As shown in Fig. 3, the CFU/mL needed for the decrease to exceed 3 times of the background (RLU detected in the absence of bacteria) differed among tested bacteria. For *Salmonella typhimurium*, less than 10^3 CFU/mL is required. On the other hand, more than 10^4 CFU/mL of *Listeria monocytogenes* and *Bacillus cereus* are required. For *Pseudomonas putida*, the required concentration is less than 10^4, but greater than 10^3. The results suggest

that the CCCP approach may be used to detect the presence of 10^3 to 10^4 viable cells for all the bacteria tested.

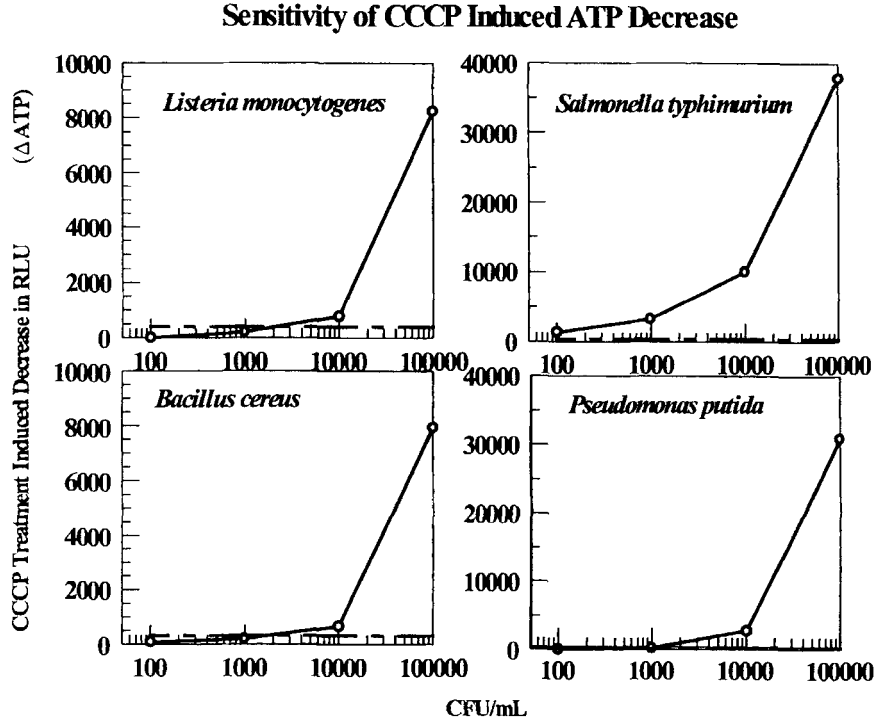

FIG. 3. SENSITIVITY OF THE CCCP TEST

Bacterial suspensions with indicated CFU/mL were used to determine the sensitivity of the CCCP test of cell viability. The difference between the two levels of ATP-related bioluminescence measured with CCCP added prior to and after cell breakage is plotted against the cell concentration. The background RLUs (without bacteria) were shown as dashed lines. The data shown represent an average of 3 independent measurements with an error range of ± 10%.

Effects of Glucose Effects on Cells After Cold Storage

Freshly cultured bacteria normally have higher level of ATP because of ample nutrient reserve in cells. Upon cold storage in TBS buffer without any nutrient supplement, cellular carbon nutrients and energy reserves are slowly consumed to support minimal metabolic activities needed for maintaining cellular viability. As shown in Fig. 4A, the cellular ATP level of tested bacteria showed decreases in cellular ATP content upon cold storage. A brief incubation with

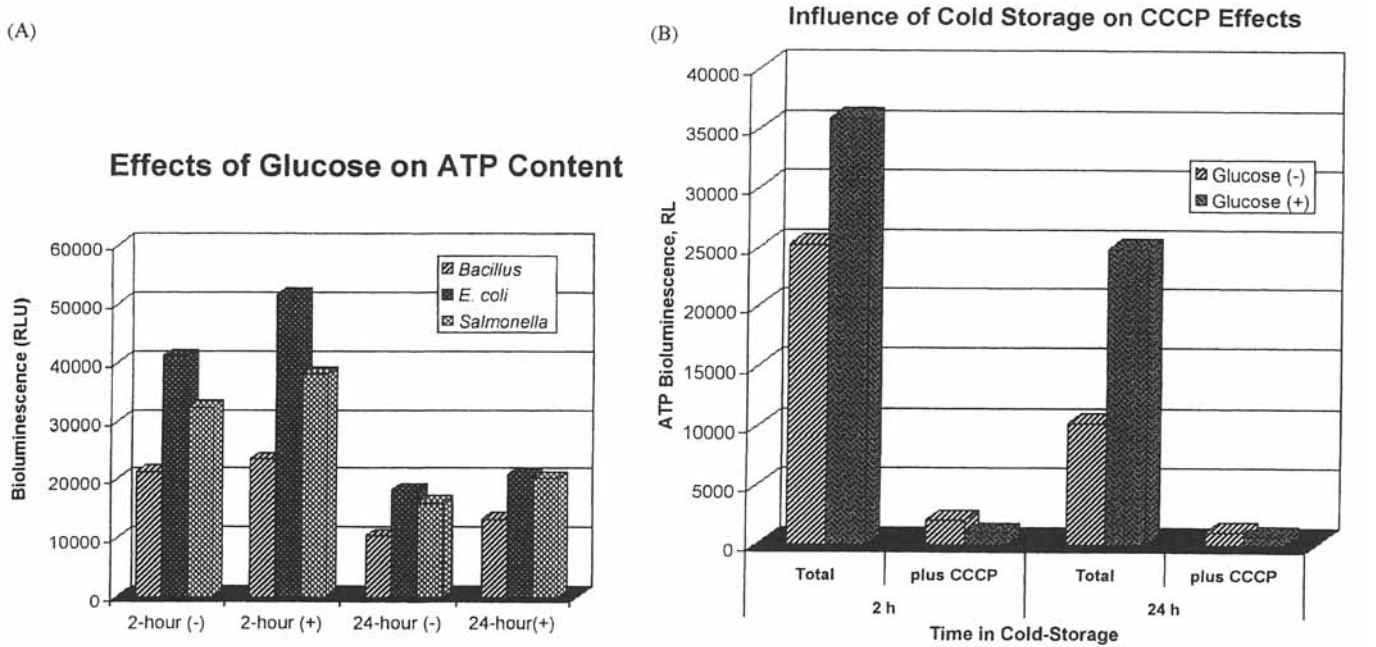

FIG. 4. (A) EFFECTS OF COLD STORAGE ON CELLULAR ATP CONTENT

Freshly harvested cultures of *Bacillus cereus, E. coli* O157:H7 and *Salmonella typhimurium* were washed and stored in buffer at 4C for about 2 and 24 h before ATP test. The bacterial samples containing 10^6 CFU were then incubated in TBS with (+) and without (-) 10 mM glucose at 37C for 10 min prior to ATP bioluminescence assay.

(B) EFFECTS OF CCCP ON CELLULAR ATP CONTENT OF COLD-STORED *LISTERIA MONOCYTOGENES*

Freshly cultured *Listeria monocytogenes* cells were kept at 4C for 2 to 24 h. ATP content of the bacteria was determined (10^6 CFU) for samples incubated at 37C for 10 min with (+) and without (-) 10 mM glucose, prior to ATP determination with and without CCCP treatment before cell breakage. The data shown represent an average of 3 independent measurements with an error range of $\pm$ 10%.

10 mM glucose at 37C significantly restored the cellular ATP level of all cold-stored bacteria. However, the extent of restoration varied from bacteria to bacteria. The observed ATP restorations indicate that glucose may be utilized by the bacteria to generate ATP. This glucose-induced ATP restoration is also sensitive to CCCP treatment. As shown in Fig. 4B, both the total and CCCP-sensitive cellular ATP levels of *Listeria monocytogenes* decreased during cold-storage. The addition of glucose substantially restored the total ATP level. However, the ATP sensitivity toward CCCP treatment was not affected with and without glucose addition. It should be mentioned that no nutrient in the TBS buffer was used for cold-storage of the bacteria, and glucose was added only 10 min prior to ATP assay. Thus, the observed increase in the ATP level may not be due to the possible cell growth during the cold storage or the brief incubation with glucose. Indeed, as determined from plate culture, the bacterial concentration (CFU/mL) remained the same during the cold storage.

CONCLUSION

To minimize the potential for outbreaks, the presence of viable pathogens in foods must be rapidly and effectively detected. To this end, we have successfully expanded our previous finding on the influence of CCCP and glucose on the ATP content of *E. coli* O157:H7 (Tu *et al.* 1999; Tu *et al.* 2000) to a number of other bacteria. Specifically, the presence of CCCP can decrease the ATP content of viable but not heat-killed cells. Upon cold storage, bacterial cells show a decrease in ATP content that may be partially restored upon brief incubation with glucose. Those findings indicate that the viability of bacteria may be ascertained by their bioenergetic responses to CCCP and glucose.

The described CCCP approach is useful for the detection of viable bacterial cells. However, the method by itself does not have any specificity toward any given pathogens. As demonstrated for the detection of viable *E. coli* O157:H7 in foods (Tu *et al.* 1999), capturing and concentrating targeted pathogen with proper immunomagnetic beads (IMB) prior to bioluminescence ATP assay may substantially enhance the specificity of described CCCP approach. Thus, proper IMB application together with CCCP approach will allow the ATP bioluminescence assay to be a rapid, sensitive and specific method for the detection of viable pathogens.

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